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TITLE OF THE INVENTION (280 characters max)

ASSAY FOR DETERMINING THE ACTIVITY OF FATTY ACID AMIDE HYDROLASE

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Respectfully submitted,

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PROVISIONAL APPLICATION FILING ONLY

Assay for Determining the Activity of Fatty Acid Amide Hydrolase

Field of the Invention

[0001] The present invention relates to methods for determining the activity of and identifying modulators of fatty acid amide hydrolases. More specifically, the invention relates to an assay, adaptable for high throughput screening, for compounds that alter fatty acid amide hydrolase activity.

Background

[0002] The identification of anandamide (N-arachidonylethanolamine, AEA) as an endogenous ligand for the cannabinoid 1 receptor (Devane *et al.* (1992) *Science* 258:1946-1949) evoked much scientific interest in the function of bioactive lipids. Other examples of endogenous ligands are oleamide (*cis*-9,10-octadecenoamide), best known for its sleep-inducing properties (Cravatt *et al.* (1995) *Science* 268:1506-1509), and 2-arachidonoylglycerol, reported to be neuroprotective after brain injury (Panikashvili *et al.* (2001) *Nature* 413:527-531).

[0003] The main mechanism for the termination of the biological activity of anandamide is hydrolysis (Giuffrida *et al.* (2001) *J. Pharmacol. Expt. Ther.* 298:7-14); the existence of an anandamide transporter has also been proposed (Compton and Martin (1997) *J. Pharmacol. Expt. Ther.* 263:1138-1143).

[0004] The enzyme responsible for the hydrolysis of anandamide, oleamide and 2-arachidonoylglycerol was cloned in 1996 and named fatty acid amide hydrolase or FAAH (Cravatt *et al.* (1996) *Nature* 384:87-87). FAAH (EC 3.5.1.4) is a membrane-bound enzyme with broad substrate specificity which is expressed in a wide variety of

human tissues and cell lines (for review see Ueda *et al.*, Chem.Phys.Lipids. 108: 107-121, (2000); Fowler *et al.*, Biochem. Pharmacol. 62: 517-526, (2001).

[0005] Inhibitors of FAAH have been predicted to potentiate the effects of the endogenous cannabinoids and thereby promote sleep, muscle relaxation and analgesia (Fowler *et al.* (2001) *Biochem. Pharmacol.* 62:517-526). Efforts to identify useful inhibitors have been hampered by the lack of simple, reproducible assays suitable for high-throughput screening. Published methods include reversed phase HPLC (9) and thin-layer chromatography (Deutsch and Chin (1993) *Biochem. Pharmacol.* 46:791-796). A fluorescence displacement method has also been described (Thumser *et al.* (1997) *Biochem. Pharmacol.* 53:433-437). An additional assay relies on extraction of the hydrolysis product with a chloroform: methanol mixture (Maurelli *et al.* (1995) *FEBS Lett.* 377:82-86), followed by counting of radioactivity. However, the toxicity of the chloroform and the cumbersome physical manipulations of this method preclude the adaptation of this assay to a high-throughput format.

[0006] There is a need in the art, therefore, for improved FAAH assays, particularly those which can be adapted to high throughput screening, for determining the activity of FAAH and for identifying modulators of FAAH.

SUMMARY OF THE INVENTION

[0007] In one aspect, the instant invention provides methods for assaying the activity and amount of fatty acid amide hydrolase (FAAH) based on differences in the physicochemical and binding properties of a FAAH substrate, and the products of its hydrolysis. For example, anandamide is hydrolyzed by FAAH to arachidonic acid and ethanolamine (Fig. 1). In brief, a substrate, for example ^3H -anandamide (ethanolamine 1 - ^3H), is incubated with a putative source of FAAH activity in a

reaction mixture. The FAAH activity, where present, catalyzes the hydrolysis of the substrate to form at least one radiolabeled hydrolysis product; the example substrate ^3H -anandamide is converted to labeled ethanolamine and unlabeled arachidonic acid. This labeled product and the labeled substrate are separated from each other and the loss of labeled substrate, or preferably, the formation of labeled product, is measured. In certain preferred embodiments, the assays are performed in parallel or in sets wherein assays conducted in the presence of compounds to be tested for their ability to modulate the FAAH activity are compared with those conducted in the absence of the compounds to be tested. In other preferred embodiments samples from patients can be assayed to determine if the FAAH activity is altered relative to a predetermined activity value.

[0008] In one aspect, the invention provides improved methods of measuring fatty acid amide hydrolase activity comprising combining a sample suspected of containing fatty acid amide hydrolase, with a labeled substrate of fatty acid amide hydrolase to form a reaction mixture; incubating the reaction mixture under conditions which allow the fatty acid amide hydrolase to hydrolyze the labeled substrate, thereby forming at least one labeled hydrolysis product; contacting the incubated reaction mixture with a selective binding material wherein the selective binding material binds either the labeled substrate or the labeled product, but not both, thereby forming a bound labeled complex; separating the bound labeled complex from the unbound labeled compound, thereby effectuating a separation of the labeled substrate from labeled product; and determining the amount of labeled substrate hydrolyzed or the amount of labeled hydrolysis product formed; thereby indicating the fatty acid amide hydrolase activity of the sample. The assay methods provided are adaptable for use in

high throughput screening systems and are contemplated to be used in drug discovery efforts.

[0009] In another aspect of the invention, methods are provided for identifying compounds which can modulate the activity of a FAAH enzyme. The methods comprise the steps of comparing the activity of a FAAH as assayed by the above method in the presence and in the absence of a test compound added to the reaction mixture; wherein a change in the activity of the fatty acid amide hydrolase indicates that the test compound modulates the activity of the fatty acid amide hydrolase. In various embodiments, the methods can be used to identify useful inhibitors or enhancers of FAAH activity. High throughput screening using the assay methods of the present invention will allow libraries of test compounds (for example, libraries produced by techniques of combinatorial chemistry) to be used in rational screening programs to identify inhibitors and enhancers of FAAH activity which are useful as candidates for drugs

[0010] In another aspect, the invention provides methods of determining altered FAAH activity in a patient. The methods comprise the steps of obtaining a sample containing cells from the patient; lysing the cells to form a cell lysate; combining the cell lysate with a labeled substrate of the fatty acid amide hydrolase, to form a reaction mixture; incubating the reaction mixture under conditions sufficient to allow a fatty acid amide hydrolase present in the cell lysate to hydrolyze the labeled substrate, thereby forming at least one labeled hydrolysis product; contacting the incubated reaction mixture with a selective binding material; wherein the selective binding material binds either the labeled substrate or a labeled hydrolysis product, but not both, thereby forming a bound labeled complex; separating the bound labeled

complex from the unbound labeled compound, thereby effectuating a separation of the labeled substrate from labeled product; determining an amount of labeled substrate hydrolyzed, or labeled hydrolysis product formed, thereby indicating the fatty acid amide hydrolase activity of the sample; and comparing the activity of the sample from the patient with a predetermined value for activity, to determine if the patient has altered fatty acid amide hydrolase activity relative to the predetermined value for activity.

[0011] These and other aspects of the present invention will be described in further detail in the Detailed Description set forth below.

[0012] **BRIEF DESCRIPTION OF THE FIGURES**

[0013] **Figure 1:** Schematic diagram of a FAAH assay. The FAAH activity in T84 membranes converts anandamide [$1\text{-}^3\text{H}$ -ethanolamine] into radiolabeled ethanolamine and unlabeled arachidonic acid. The labeled anandamide and unlabeled arachidonic acid are selectively bound to charcoal in filterplates, whereas the radiolabeled ethanolamine is collected in the flow-through and counted.

[0014] **Figure 2** depicts an embodiment of a FAAH assay method. The reaction takes place in a reaction plate at room temperature. After 60 minutes, 60 μl of the reaction mixture is transferred to a charcoal-filled filter plate. The filter plate is fitted on top of a Dynex plate; the assembly is then centrifuged for 5 minutes at 2000 rpm.

[0015] **Figure 3:** Arachidonic acid and anandamide, but not ethanolamine, bind to activated charcoal. Four different radiolabeled tracers were used: anandamide [$1\text{-}^3\text{H}$ -ethanolamine], anandamide [arachidonyl-5,6,8,9,11,12,14,15- ^3H], arachidonic acid [5,6,8,9,11,12,14,15- ^3H (N)] and ethanolamine ([2- ^{14}C] ethan-1-ol-2-amine hydrochloride). The tracers were incubated with membranes prepared from mouse

liver, T84 cells, HeLa cells or with vehicle, for 60 minutes at room temperature. The percentage (average of triplicate determinations \pm s.e.m.) of total radioactivity recovered in the flow-through is shown.

[0016] Figures 4A-4B: Characterization of a preferred assay. A: Time-course of hydrolysis of ^3H -anandamide by FAAH at room temperature and at 37°C . The average of triplicate determinations \pm s.e.m. is shown. B: Determination of K_m for FAAH. T84 cell membranes (3.5 μg protein per well) were incubated with a range of concentrations of ^3H -anandamide. The reactions were carried out at room temperature in the presence or absence of inhibitors (either oleyl trimethylfluoro ketone or MAFP). The experiment where oleyl trimethylfluoro ketone was used to determine nonspecific binding is shown; the results with MAFP were comparable. Each point reflects the average of triplicate determinations \pm s.e.m.

[0017] DETAILED DESCRIPTION OF THE INVENTION

[0018] The reference works, patents, patent applications, and scientific literature, including accession numbers to GenBank database sequences, that are referred to herein are hereby incorporated by reference in their entireties.

[0019] Various definitions are made throughout this document. Most words have the meaning that would be attributed to those words by one skilled in the art. Words specifically defined either below or elsewhere in this document have the meaning provided in the context of the present invention as a whole and as are typically understood by those skilled in the art. Any conflict between an art-understood definition of a word or phrase and a definition of the word or phrase as specifically taught in this specification shall be resolved in favor of the latter.

[0020] Standard reference works setting forth the general principles of recombinant DNA technology known to those of skill in the art include Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York (2002); Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, New York (2001); Kaufman *et al.*, Eds., Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton (1995).

[0021] As used herein, a "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioisotopes and fluorescent labels. Examples of radioisotopes that may be used in the method of the invention include ^3H and ^{14}C .

[0022] As used herein, "purified" refers to at least partial separation of a molecule from other molecules with which it is normally associated. For example, a purified protein is a protein that is at least partially separated from other cellular material with which it is normally associated.

[0023] As used herein, the term "murine" means originating in a member of the family *Muridae*. A murine FAAH preferably originates in a mouse, or rat.

[0024] In a first aspect the invention provides methods of assaying a fatty acid amide hydrolase (FAAH). In a presently preferred embodiment, the method of assay comprises the steps of combining a sample suspected of containing a FAAH with a labeled substrate of FAAH to form a reaction; incubating the reaction mixture under conditions sufficient to allow the fatty acid amide hydrolase to hydrolyze the labeled substrate, forming one or more labeled hydrolysis products; contacting the incubated reaction mixture with a selective binding material; wherein the selective binding material binds either the labeled substrate or a labeled hydrolysis product, but not

both, to form a bound labeled complex; separating the bound labeled complex from the unbound labeled compound, thereby effectuating a separation of the labeled substrate from labeled product; and determining an amount of labeled substrate hydrolyzed, or labeled hydrolysis product formed, thereby indicating the fatty acid amide hydrolase activity of the sample.

[0025] The sample, in preferred embodiments, is a biological sample, or a sample comprising biological material, in particular biological membranes. In other preferred embodiments, the sample is from a purification step in the purification of FAAH from a biological source. The sample comprises biological membranes or portions thereof, or comprises lipid bilayers, or artificial membrane systems, monolayers, vesicles or micelles. A sample in some embodiments comprises a purified or recombinant FAAH reconstituted into a phospholipid-containing reaction mixture.

[0026] The substrate may be any substrate, putative substrate, or substrate analog of FAAH. The assay is also adapted for use to determine the utility of a compound as a substrate of FAAH. Substrates preferred for use in various embodiments of the present invention include, but are not limited to endocannabinoids or analogs thereof, fatty acid ethanolamides or analogs thereof, fatty acid primary amides or analogs thereof, and analogs of any of the foregoing labeled with a detectable label. Presently preferred substrates of particular interest include, for example, anandamide, oleamide, and 2-arachidonoylglycerol.

[0027] The substrate may be radioisotopically labeled in any manner known in the art for labeling compounds for detection. Presently preferred isotopes, such as ^3H or ^{14}C , are readily detected via liquid scintillation counting and can facilitate adaptation of the assays for high throughput drug screening. Synthetic substrates so labeled are readily available commercially or can be synthesized.

[0028] Other labels such as fluorescent labels capable of detection, for example fluorimetric detection, are also readily adapted to high throughput screening. Preferred fluorescent labels are detectable in biological systems with both proteins and nucleic acids present, therefore preferred labels when assaying cruder cell lysates and homogenates possess both excitation and emission optima which are not masked by these other biological components. In one embodiment the unhydrolyzed labeled substrate has identical fluorescent properties with the hydrolysis product. It is also possible to design substrates wherein hydrolysis results in a change in fluorescence, for example, where a dye-dye interaction in the unhydrolyzed molecule is required to maintain a ground state. Such substrates are useful in conjunction with the present invention. Colorimetric labels are also contemplated for use herein. Generally, appropriate labels are those which do not alter the substrate's susceptibility to hydrolysis by the enzyme and for which detection systems have been developed.

[0029] Preferably the substrate is present at concentrations at vast excess relative to the concentration of the enzyme. Under such conditions classical enzyme kinetics can be used to determine a rate constant (K_m) and a velocity (V_{max}) of the reaction. Kinetic studies are useful for mechanistic studies; they are also powerful tools for evaluating inhibitors (see below). Standard texts directed at those skilled in the art of enzyme kinetics such as Segel, I.H., *Enzyme Kinetics : Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (1993, Wiley-Interscience, ISBN 0471303097) provide complete guidance to establishing these parameters and demonstrate the use, for example, of Lineweaver-Burke plots as graphical tools to simplify the determination of the kinetic parameters.

[0030] High throughput screening systems are known in the art. Such systems often involve the use of multiwell plates to increase the number of assays conducted

simultaneously from a few to a hundred, a few hundred or even a few thousand. Presently preferred high throughput screening adaptations of the methods of the present invention provide capability of screening about one hundred to about one or more thousand assays in a short time. Presently preferred high throughput screening systems include robotic components for example for sample handling, dispensing, reagent addition, and other functions to improve accuracy and eliminate the labor intensive aspects of large numbers of assays. Detection systems for high throughput screening programs are known to those of ordinary skill in the art. Preferred detection systems include, but are not limited to, scintillation counting (including, for example, solid and liquid scintillation for counting gamma or beta particles, or luminescent samples, filter counting, Cerenkov counting, and scintillating microplate counting), fluorescence detection (including, for example, intensity, fluorescence polarization, time-resolved fluorescence, fluorescence resonance energy transfer (FRET)), luminescence, and absorbance.

[0031] In a presently preferred embodiment, to achieve high-throughput screening, samples are placed on a multicontainer carrier or platform. A multicontainer carrier facilitates measuring reactions of a plurality of candidate compounds simultaneously. For illustration purposes, but not by way of limitation, a multi-well microplate, such as a 96 or a 384 well microplate, that can accommodate 96 or 384 different test reactions, is used as the carrier. Such multi-well microplates, and methods for their use in numerous assays, are both known in the art and commercially available through sources such as Sigma Chemical Co., BIOCHEMICAL ORGANIC COMPOUND AND DIAGNOSTIC REAGENTS, 2002, pages 2495-2511.

[0032] The methods of the present invention are adaptable to miniaturization techniques. Assays for purposes of high throughput screening are often conducted in

small volumes. Procedures are currently known to those of skill in the art of reducing volumes of assays in a variety of ways. Microfluidics and microcapillary methodologies now enable assays to be performed down to nanoliter quantities. It is to be understood that the basic principles of the methods and assays apply notwithstanding the volume of the assay, or the manner in which the materials are measured or transported.

[0033] The process of determining incubation conditions is routine in the art. Preferred incubation conditions relate, for example, to enzyme stability. Temperature optimums are routine to determine given the specification of the assay and should be determined for a given enzyme and substrate combination for optimum results. Times and other conditions for incubation involve likewise routine determinations. Preference is given to those conditions which result in linearity of the assay. Presently preferred temperatures and times include room temperature for 1 hour, or 37 °C for 30 min, using FAAH from a variety of sources and using anadamide as a substrate. Standard texts directed at those skilled in the art of enzyme assays such as Segel, *supra*, provide complete guidance to optimizing assays, including the incubation conditions. Further characterization of the preferred embodiments is provided in the working examples.

[0034] The methods also comprise a step wherein the incubated reaction mixture is contacted with a selective binding material. It is important to note that the selective binding material preferred for this assay may vary with the substrate selected. The selective binding materials comprise materials which can readily be separated from the bulk reaction mixture by separation techniques which are known in the art. Effective separations can be based on differences in particle size, density, composition and magnetic susceptibility. Material is separated from a reaction

mixture by, for example, gravity settling, filtration (including, for example, membrane separations), centrifugation, A presently preferred method of contacting the incubated reaction mixture with a selective binding material comprises activated charcoal.

[0035] Where the substrate is anandamide, for example, activated charcoal binds, through adsorption, the substrate and one of the hydrolysis products, arachidonic acid. The other hydrolysis product, ethanolamine, does not adsorb to the activated charcoal. For preferred embodiments, the anandamide substrate is labeled on the ethanolamine moiety. The FAAH hydrolyzes the labeled anandamide into labeled ethanolamine and unlabeled arachidonic acid. After adsorption of the labeled substrate and the unlabeled arachidonic acid to the charcoal, the labeled ethanolamine in solution can be separated from the bound labeled substrate through a simple filtration step. This facilitates the measurement of product without interference from substrate. By removing some compounds through the binding process, the sample is less likely to interfere, for example by quenching, with the measurement of the labeled product. Existing technology has already been adapted for filtering large numbers of samples simultaneously, for example in multiwell filters. This attribute also is adaptable therefore to high throughput screening programs.

[0036] Although it is often preferred to measure the formation of product in the methods of this invention, assay conditions and selective binding material may be selected wherein the disappearance of substrate is measured. This indirect method is adaptable particularly where a partially purified or substantially purified enzyme is used, or where it is known that there is only one route by which substrate disappears from the reaction mixture. It is also preferred to measure substrate disappearance wherein a selective binding material which binds the substrate is not readily available. This can be useful in particular in embodiments where the substrate cannot

practicably be bound to effect its separation from free hydrolysis product but where the hydrolysis product can be more readily bound to effectively separate it from the free labeled substrate. This gives a practitioner the flexibility to choose from a broader range of selective binding materials.

[0037] The determination of the amount of labeled substrate hydrolyzed or the labeled hydrolysis product formed is preferably a direct method of quantitating the amount of label present. As discussed above, a variety of detection techniques are suitable for the present invention, and an appropriate detection method relates to the properties of the label present on the substrate or the product. Presently preferred for use with the methods of the invention are radioisotopically labeled substrates and fluorescently labeled substrates, quantified respectively by scintillation counting and fluorescence detection, respectively. In one preferred embodiment, the substrate is ^3H -anandamide, the detection is of the product, ^3H -ethanolamine, formed, and the detection is with a liquid scintillation counting plate reader from the filtrate of an assay conducted in a multiwell plate.

[0038] In another aspect of the invention, methods are provided for identifying modulators of fatty acid amide hydrolase. The methods comprise comparing the activity of fatty acid amide hydrolase as assayed by the method as described herein above, in the presence and in the absence of a test compound added to the reaction mixture; a change in the activity of the fatty acid amide hydrolase indicates that the test compound modulates the activity of the fatty acid amide hydrolase.

[0039] In preferred embodiments, the modulator to be identified is part of compound library, for example, a library of compounds formed by combinatorial chemistry, or a library of related compounds identified or synthesized as part of a research program. The putative modulators (i.e the compounds to be tested, test

compounds) are preferably a small molecule with properties that would allow it to have utility for pharmaceutical compositions. Preferred modulators have low nonspecific toxicity, and high specificity for modulating FAAH.

[0040] Modulators include inhibitors of FAAH activity and activators of FAAH.

Assay parameters such as concentration of substrate, incubation conditions, and concentration of compounds to be tested may be varied to more fully appreciate the modulation effects of a test compound.

[0041] Preferred modulators that are inhibitors can be identified by their ability to decrease the activity of FAAH relative to that of a control reaction mixture lacking the test compound. Preferably, reaction volumes are maintained constant by adjusting the volume to compensate for the addition of a test compound where the addition of a test compound alters the reaction volume. Most preferably any difference in volume is compensated for by a corresponding addition of a volume, equal to the difference, of the vehicle in which the test compounds are dissolved.

[0042] In one aspect of the invention, the modulator identified is an inhibitor of the FAAH activity. Enzyme inhibitors include reversible and irreversible inhibitors. Preferred inhibitors in some embodiments are reversible inhibitors. Reversible inhibitors of FAAH include competitive inhibitors, which raise the apparent K_m of the reaction, noncompetitive inhibitors, which reduce the V_{max} of the enzyme, and inhibitors which affect both K_m and V_{max} , namely, mixed inhibitors and uncompetitive inhibitors. The type of inhibitor identified in screens can be determined through the use of kinetic assay studies according to the methods of the present invention. For the purposes of the present invention, however, any compound which decreases the apparent activity of the enzyme is considered a modulator of the inhibitor type. In

other preferred embodiments inhibiting modulators include irreversible inhibitors as well as noncompetitive inhibitors, whose action may be irreversible.

[0043] In preferred embodiments the test compound inhibits the FAAH reproducibly, as determined by a statistically significant difference by an appropriate statistical test. Such tests are known to those of skill in the art of statistical determinations in scientific measurements, such as those of biological or biochemical systems. In some embodiments, the test compound inhibits fatty acid amide hydrolase activity about 1%, or at least about 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, or about 95% or more, or up to 100%. In other preferred embodiments, the inhibitor statistically significantly increases the apparent K_m or decreases the V_{max} of the reaction.

[0044] In another aspect of the invention, the test compound increases fatty acid amide hydrolase activity. Various types of enzyme activators are known to those of skill in the art. Some activators are known to activate enzyme reactions by increasing the velocity of the reaction, others are known to alter the equilibrium attained.

Activators that combine reversibly with enzyme reaction components (for example, the enzyme, the substrate, or enzyme-substrate or enzyme-product intermediates) to increase the velocity of the reaction are presently preferred. Nonspecific activators may also increase the reaction rate. For purposes of the present invention, any compound that increases the apparent activity of the enzyme is considered a modulator of the activator type. For example many enzymes are known to have increased activity in the presence of certain anions. Other enzymes, in particular those acting on water-insoluble and charge-neutral molecules, are activated by negatively charged lipophilic molecules. Some membrane bound enzymes are known to be activated, for example, by specific phospholipids.

[0045] In preferred embodiments the test compound activates the FAAH reproducibly, as determined by a statistically significant difference by an appropriate statistical test. Such tests are known to those of skill in the art of statistical determinations in scientific measurements, such as those of biological or biochemical systems. In some embodiments, fatty acid amide hydrolase activity is increased about 1%, or at least about 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, or about 95% or more, or up to 100%. In other embodiments, activation is 10-30%, 30-50%, 50-70%, 70-90%, 90-110%. In some embodiments, the activator increases FAAH activity 100-300%, 300-500%, 500-700%, or 700-900% or more.

[0046] In certain preferred embodiments of the methods of the invention, the fatty acid amide hydrolase is a crude cell lysate or a cell homogenate. In some embodiments the FAAH is isolated partially or substantially from cells. In other embodiments, the fatty acid amide hydrolase is recombinantly produced. The fatty acid amide hydrolase may be from any biological source including mammalian fatty acid amide hydrolases from such animals as pigs, rodents (including rats and mice), and humans, or it may be recombinant FAAH produced in any organism known to be useful for the production of recombinant proteins. Synthetic FAAH based on a particular known amino acid sequence, or on a consensus or combination of any number of sequences that result in an active FAAH is also contemplated for use herein.

[0047] Examples of known amino acid sequences of fatty acid amide hydrolases from various biological sources include, for example, SEQ ID NOS: 2, 4, 6, and 8. Examples of known nucleic acid sequences encoding fatty acid amide hydrolases include SEQ ID NOS: 1, 3, 5 and 7. Persons of ordinary skill in the art will recognize that such sequences and the modifications thereof which retain activity, can be used in

accordance with the techniques found in references such as those provided above to generate, for example, biological FAAH, recombinant FAAH, overproduced FAAH, or genetically modified FAAH. It is contemplated herein that any form of FAAH is adapted for assay with the methods of the present invention.

[0048] In a preferred embodiment the assay is used to measure the activity and identify modulators of FAAH with altered amino acid sequences. Such altered FAAH can result from mutations in the genes which encode FAAH enzymes. In one embodiment, the assay is used to study the activity of, and identify modulators of, altered FAAH in connection with cannabis abuse. For example, a recent paper by Sipe *et al.* (PNAS 99:8394-8398, 2002) describes a strong association between the substitution of Thr for Pro at position 129, and substance abuse in humans. The use of the assay of the present invention may help identify FAAH from humans with higher specific activities or higher levels of net activity, thereby resulting in reduced concentrations of endocannabinoids in the brain, thereby leading to a tendency to seek external cannabinoids to compensate.

[0049] In another aspect, the invention provides methods of determining altered FAAH activity in a patient. The methods comprise the steps of obtaining a sample containing cells from the patient; lysing the cells to form a cell lysate; combining the cell lysate with a labeled substrate of the fatty acid amide hydrolase, to form a reaction mixture; incubating the reaction mixture under conditions sufficient to allow a fatty acid amide hydrolase present in the cell lysate to hydrolyze the labeled substrate, thereby forming at least one labeled hydrolysis product; contacting the incubated reaction mixture with a selective binding material; wherein the selective binding material binds either the labeled substrate or a labeled hydrolysis product, but not both, thereby forming a bound labeled complex; separating the bound labeled

complex from the unbound labeled compound, thereby effectuating a separation of the labeled substrate from labeled product; determining an amount of labeled substrate hydrolyzed, or labeled hydrolysis product formed, thereby indicating the fatty acid amide hydrolase activity of the sample; and comparing the activity of the sample from the patient with a predetermined value for activity, to determine if the patient has altered fatty acid amide hydrolase activity relative to the predetermined value for activity.

[0050] Preferably, the fatty acid amide hydrolase activity from a sample of fluid or tissue originating from the patient is measured. Presently preferred are blood, lymph or tissue samples. In particularly preferred embodiments, the sample is from lymphocytes from the patient. In preferred embodiments, where the sample is from a woman, the results enable a physician to screen pregnant women for risk of miscarriage (spontaneous abortion) or to screen women seeking fertility treatment for risk of failure of *in vitro* fertilization procedures. In preferred embodiments, results from the patient's sample are compared to results from samples of normal individuals, particularly those comparably positioned in terms of demographic criteria. Results of the assays of the present method may be used to determine either or both of altered specific activity (FAAH activity per unit of FAAH mass) or altered total FAAH activity (net amount of product formed per time). For example, increases in activity can be due to increases in specific activity of the FAAH, for example by alteration of the active site via amino acid alteration, gene mutation and the like, or through alteration of the total amount of FAAH protein present, without a change in the specific activity.

[0051] In the methods of the invention, the fatty acid amide hydrolase may be substantially purified from the lymphocyte cell lysate. FAAH may be derived from

any source, such that the FAAH retains the potential for FAAH activity. For example, FAAH may be purified from cells expressing FAAH, FAAH may be produced in a recombinant system, including, but not limited to bacterial cells, yeast cells, insect cells, and mammalian cells. FAAH may be derived from any organism that produces FAAH. For example, FAAH may be derived from mammalian cells such as human cells, rodent cells (*e.g.*, mouse and rat cells), or from any other organism that produces FAAH that has FAAH activity. Recombinant FAAH may be produced using techniques known in the art with any nucleic acid sequence encoding FAAH. Examples of cloned FAAHs include various mammalian FAAH such as, pig FAAH, rodent FAAH (*e.g.*, mouse FAAH and rat FAAH), and human FAAH. DNA sequences encoding FAAH and polypeptide sequences of various FAAHs are included in the appended Sequence Listing.

[0052] FAAH may be purified by standard methods in the art. The FAAH preparation may be in association with cell membranes or artificial membranes, or may be substantially free of cellular material.

[0053] Throughout the specification, reference is made to certain publications and patents. The entireties of each of these references is incorporated herein and forms a part of this disclosure.

[0054] EXAMPLES

[0055] Example 1

[0056] Radiolabeled anandamide [1-³H-ethanolamine] was obtained from American Radiolabeled Chemicals (10-20 Ci/mmol, catalog number ARC-626; St.

originally done at 37°C for 30 min; but subsequent experiments indicated that the enzyme displayed good activity at room temperature, and experiments were performed at room temperature for 60 minutes unless otherwise indicated.

[0059] During the one hour incubation, 96-well Multiscreen filter plates (catalog number MAFCNOB50; Millipore, Bedford, Massachusetts, USA) were loaded with 25 µl activated charcoal (Multiscreen column loader, catalog number MACL09625, Millipore) and washed once with 100 µl methanol. Also during the incubation, 96-well DYNEX MicroLite plates (catalog number NL510410) were loaded with 100 µl MicroScint40 (catalog number 6013641, Packard Bioscience, Meriden, Connecticut, USA). After the one hour incubation, 60 µl of the reaction mix was transferred to the charcoal plates, which were then assembled on top of the DYNEX plates using Centrifuge Alignment Frames (catalog number MACF09604, Millipore). The unbound labeled ethanolamine was centrifuged through to the bottom plate (5 min at 2,000 rpm), which was preloaded with the scintillant, as described above. The plates were sealed and left at room temperature for 1 hour before counting on a Hewlett Packard TopCount. For determination of K_m values, 1 µM ^3H -AEA was combined with 30 µM unlabeled AEA and serial 2-fold dilutions were made.

[0060] Uncleaved ^3H -anandamide, as well as the unlabeled arachidonic acid, is absorbed by the charcoal. In contrast, the labeled ^3H - ethanolamine flows through the charcoal mini-columns into 96 well counting plates when a vacuum or centrifugation is applied. The 96 well plates can then be read on a Hewlett Packard TopCount (Fig.2.)

[0061] *Charcoal selectively binds anandamide and arachidonic acid, but not ethanolamine:*

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[0061] *Charcoal selectively binds anandamide and arachidonic acid, but not ethanolamine:*

[0062] Four different radioactive tracers were used to explore the binding characteristics of the charcoal used in the assay: two tritiated forms of anandamide (labeled either on the ethanolamine moiety; anandamide [$1\text{-}^3\text{H}$ -ethanolamine], or on the arachidonic acid moiety; arachidonic acid [$5,6,8,9,11,12,14,15\text{-}^3\text{H}$ (N)]), as well as tritiated arachidonic acid, and ^{14}C -labeled ethanolamine. Specific amounts of these tracers were incubated with membrane preparations, added to the pre-washed charcoal and recovered by centrifugation, as described in the methods. The recovered radioactivity was counted and expressed as percentage of the amount added. As shown in Fig. 3, when no membranes were present, *i.e.*, there was no FAAH-mediated conversion of anandamide to arachidonic acid plus ethanolamine, neither of the two labeled forms of anandamide could be detected in the flow-through, indicating that the tracers were bound to the charcoal. Tritiated arachidonic acid was also absorbed onto the charcoal and could not be detected in the flowthrough. Of the four tracers used, only the ^{14}C -ethanolamine could be recovered.

[0063] It is known that HeLa cells, a human carcinoma cell line, do not express FAAH (Ueda *et al.* (2000) *Chem.Phys.Lipids*. 108:107-121). Therefore, this cell line was used as a negative control, and to investigate the possible contribution of non-FAAH enzymes to the hydrolysis of anandamide. There was essentially no recovery of radioactivity after incubation of ^3H -anandamide with HeLa cell membranes, confirming the absence of FAAH (Fig. 3).

[0064] When the tracers were incubated with membranes prepared from mouse liver or T84 cells, good recovery of radioactivity was found for the anandamide labeled on the N-terminus of the amide (^3H -ethanolamine), but not for the anandamide carrying the tritium label on the hydrocarbon chain of the fatty acid (^3H -AA). All experiments consistently showed that anandamide and arachidonic acid, in

contrast to ethanolamine, were absorbed onto the charcoal column. Therefore, when radiolabeled anandamide is incubated with a cell lysate containing FAAH the radioactivity recovered in the flow-through must be attributed to the radiolabeled ethanolamine. This experiment indicates that it is possible to separate anandamide and ethanolamine using charcoal. Other products, such as SAX and C18 resin were also tested, but were less effective (not shown).

[0065] *Characterization of the FAAH assay:*

[0066] FAAH activity is found in a large variety of cells. In rodents, the liver, followed by the brain, seems to have the highest expression of FAAH, whereas in humans, the expression is highest in the pancreas and brain, but lower in the liver (Ueda *et al.* (2000) *Chem.Phys.Lipids.* 108:107-121). The expression of human FAAH mRNA in T84 cells was found by expression profiling using DNA microarrays (not shown) and confirmed by subsequent experiments.

[0067] The signal output of the assay was determined to be linear in the range of 0.42-3.5 $\mu\text{g}/\text{well}$ T84 membrane. Since an embodiment of the invention is a HTS-compatible assay, it was verified that the reaction was linear over more than 1 hour at the amount of protein used. As shown in Figure 4 A, the rate of the reaction at room temperature was linear over a period of 90 minutes when 3.5 $\mu\text{g}/\text{well}$ protein was used. This experiment was performed both at room temperature and at 37°C, with the enzyme being slightly more active at the higher temperature, as expected. For the performance of HTS assays, an incubation period of 1 hour at room temperature and a protein amount of 3.5 $\mu\text{g}/\text{well}$ was chosen based on these results.

[0068] *Kinetic analysis of FAAH activity:*

[0069] Kinetic analyses of the hydrolysis of anandamide by FAAH in T84 human colorectal carcinoma cells demonstrated a K_m of $1.1 \pm 0.17 \mu M$. (Fig. 4 B). This is close to the value that has been reported for human brain ($2 \mu M$, see Maccarrone *et al.* (Maccarrone *et al.* (1999) *Anal. Biochem.* 267:314-318)). Overall, the reported K_m values in the literature for FAAH reactions range widely, from 0.8 to $80 \mu M$, depending on the tissue, species and method used (Fowler *et al.* (2001) *Biochem. Pharmacol.* 62:517-526). For instance, Desarnaud *et al.* (Desarnaud *et al.* (1995) *J. Biol. Chem.* 270, 6030-6035) found a K_m of $12.7 \mu M$ using rat brain microsomes. In the N18 mouse neuroblastoma cell line, the K_m was determined to be $9.0 \mu M$ (Maurelli *et al.* (1995) *FEBS Lett.* 377:82-86). Apart from species differences, the variability in these values has been ascribed to the observation that both the substrate and product of this enzyme can form micelles, which may affect the enzyme activity (Fowler *et al.* (2001) *Biochem. Pharmacol.* 62:517-526).

[0070] *Validation of the FAAH assay using reference compounds:*

[0071] Several of the FAAH inhibitors that have been described in the literature were tested in the assay to validate its ability to identify inhibitors. Of the inhibitors that are commercially available, methyl arachidonyl fluorophosphate (MAFP), which is also an inhibitor of cytosolic phospholipase A_2 , is the most potent, with reported IC_{50} values of $1-3 nM$ (Ueda *et al.* (2000) *Chem. Phys. Lipids* 108, 107-121; Deutsch *et al.* (1997) *Biochem. Pharmacol.* 53, 255-260). Its potency as an inhibitor of FAAH was confirmed with the determination of an IC_{50} value of $0.8 nM$.

[0072] Oleyl trifluoromethyl ketone, a transition-state inhibitor, has also been reported to be a potent inhibitor of FAAH, with an IC_{50} of $73.3 nM$ in the studies here

presented, it was in the same range as the IC₅₀ values of 28 - 41 nM found by Tiger *et al.* (2000; *Biochem. Pharmacol.* 59, 647-653).

[0073] Phenylmethanesulfonyl fluoride, an inhibitor of serine proteases, was shown to be an inhibitor of FAAH, although with a low potency. Its pIC₅₀ for the inhibition of rat brain FAAH in another group was between 5.92 and 4.16, depending on the pH (Holt *et al.* (2001) *Br. J. Pharmacol.* 133, 513-520). At a concentration of 100 µM, it abolished anandamide hydrolysis in N18 mouse neuroblastoma cells (Maurelli *et al.* (1995) *FEBS Lett.* 377, 82-86). It was used at a concentration of 1.5 mM by Deutsch *et al.* (1993; *Biochem. Pharmacol.* 46, 791-796) to block FAAH activity in neuroblastoma and glial cells. At 25 µM, it inhibited the hydrolysis of rat brain microsomal anandamide by 48 % (Desarnaud *et al.* (1995) *J. Biol. Chem.*, 270, 6030-6035). The IC₅₀ value (16 µM) obtained in this work thus corresponds well to the numbers reported in the literature.

[0074] Finally, product inhibition was tested by using the FAAH products oleic acid and arachidonic acid. Maurelli *et al.* (1995; *FEBS Lett.* 377, 82-86) found that 100 µM anandamide abolished the activity of FAAH. In this work, these compounds inhibited FAAH activity with IC₅₀ values in the low micromolar range (Table 1).

[0075] These results confirm that the assay provides a robust method for the evaluation of FAAH activity. The assay was successfully validated using reference inhibitors. As the assay may be performed simultaneously in multiple reactions, such as in a 96 well plate, for example, the assay is adaptable for high-throughput screening of compound collections as well as natural product or combinatorial libraries.

Table 1. Activity of known FAAH inhibitors in T84 membranes

COMPOUND	IC ₅₀ (nM)	Literature (ref)
Methyl arachidonyl fluorophosphate	0.8 ± 0.7	2.5 ⁽¹⁾ 1-3 ⁽²⁾
Oleyl trifluoromethyl ketone	73.3 ± 19.8	39 ⁽³⁾ 24-41 ⁽⁴⁾
Phenylmethylsulfonyl fluoride	15978 ± 8863	1200-69000 ⁽⁵⁾
Arachidonic acid	931.4 ± 255.4	-
Oleic acid	1936 ± 401	-

The average ± S.E. of values obtained in 2-5 experiments, each done in triplicate, is given. The numbers in the column on the right refer to literature data. References : (1) : Deutsch *et al.* Biochem. Pharmacol. 1997, 53: 255-260. (2) : Ueda *et al.* Chem. Phys.Lipids 2000, 108 : 107-121. (3) Fowler *et al.* Br. J. Pharmacol. 2000, 131: 498-504. (4) Tiger *et al.* Biochem. Pharmacol. 2000, 59:647-653. (5) Holt *et al.* Br. J. Pharmacol. 2001, 133: 513-520.

The foregoing description, examples and accompanying figures generally describe the invention. The description is for purposes of providing illustrations; the present invention is not to be limited by the specific embodiments described herein. One of skill in the art will appreciate that various modifications of the invention may be made in addition to those described herein. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method of assaying the activity of a fatty acid amide hydrolase comprising the steps of:
 - combining a sample suspected of containing a fatty acid amide hydrolase, with a labeled substrate of the fatty acid amide hydrolase, to form a reaction mixture;
 - incubating the reaction mixture under conditions sufficient to allow the fatty acid amide hydrolase to hydrolyze the labeled substrate, thereby forming at least one labeled hydrolysis product;
 - contacting the incubated reaction mixture with a selective binding material; wherein the selective binding material binds either the labeled substrate or a labeled hydrolysis product, but not both, thereby forming a bound labeled complex;
 - separating the bound labeled complex from the incubated reaction mixture;
 - and
 - determining an amount of labeled substrate hydrolyzed, or labeled hydrolysis product formed, thereby indicating the fatty acid amide hydrolase activity of the sample.
2. The method of claim 1 wherein the sample comprises biological membranes, lipid bilayers, or micelles.
3. The method of claim 1 wherein the substrate is an endocannabinoid, a fatty acid ethanolamide or a fatty acid primary amide, or an analog of the foregoing.
4. The method of claim 1 wherein the substrate is anandamide.

5. The method of claim 1 wherein the substrate is oleamide.
6. The method of claim 1 wherein the substrate is 2-arachidonoylglycerol.
7. The method of claim 1 wherein the substrate is labeled with a radioisotope.
8. The method of claim 7 wherein the radioisotope is ^3H or ^{14}C .
9. The method of claim 1 wherein the substrate is labeled with a fluorescent label.
10. The method of claim 1 wherein the selective binding material comprises carbon.
11. The method of claim 8 wherein the selective binding material is activated charcoal.
12. The method of claim 9 wherein the activated charcoal comprises a filter.
13. The method of claim 1 wherein the selective binding material binds the labeled substrate but not the labeled product.
14. The method of claim 1 wherein the separating step comprises filtration, gravity settling or centrifugation.

15. The method of claim 1 wherein the determining step is performed via liquid scintillation counting or by measurement of fluorescence energy.
16. The method of claim 1 conducted in a multiwell plate.
17. The method of claim 1 comprising at least a portion of a high throughput screening program.
18. The method of claim 1 wherein the method is conducted in conjunction with a drug discovery effort.
19. A method of identifying a compound that modulates the activity of a fatty acid amide hydrolase comprising the steps of:
 - comparing the activity of a fatty acid amide hydrolase as assayed by the method of claim 1, in the presence and in the absence of a test compound added to the reaction mixture;
 - wherein a change in the activity of the fatty acid amide hydrolase indicates that the test compound modulates the activity of the fatty acid amide hydrolase.
20. The method of claim 19 wherein the test compound is selected from a library of compounds.
21. The method of claim 19 wherein the test compound inhibits the activity of the fatty acid amide hydrolase activity.

22. The method of claim 21 wherein said fatty acid amide hydrolase activity is inhibited at least about 5%.
23. The method of claim 21 wherein said fatty acid amide hydrolase activity is inhibited at least about 20%.
24. The method of claim 21 wherein said fatty acid amide hydrolase activity is inhibited at least about 50%.
25. The method of claim 21 wherein said fatty acid amide hydrolase activity is inhibited at least about 80%.
26. The method of claim 21 wherein said fatty acid amide hydrolase activity is inhibited at least about 95% or more.
27. The method of claim 21 wherein said test compound increases said fatty acid amide hydrolase activity.
28. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased at least about 5%.
29. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased at least about 30%.
30. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased at least about 50%.

31. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased at least about 70%.
32. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased at least about 100%.
33. The method of claim 27 wherein said fatty acid amide hydrolase activity is increased between about two-fold to ten-fold.
34. The method of claim 19, which comprises the use of a multi-well plate.
35. The method of claim 19 conducted in a multiwell plate.
36. The method of claim 19 comprising at least a portion of a high throughput screening.
37. The method of claim 19 wherein the method is conducted in conjugation with a drug discovery effort.
38. The method of claim 1 wherein said fatty acid amide hydrolase is a mammalian fatty acid amide hydrolase.
39. The method of claim 1 wherein said fatty acid amide hydrolase is a porcine fatty acid amide hydrolase.

40. The method of claim 1 wherein said fatty acid amide hydrolase is a rodent fatty acid amide hydrolase.

41. The method of claim 1 wherein said fatty acid amide hydrolase is a murine fatty acid amide hydrolase.

42. The method of claim 1 wherein said fatty acid amide hydrolase is a rat fatty acid amide hydrolase.

43. The method of claim 1 wherein said fatty acid amide hydrolase is a mouse fatty acid amide hydrolase.

44. The method of claim 1 wherein said fatty acid amide hydrolase is a human fatty acid amide hydrolase.

45. A method for determining altered fatty acid amide hydrolase activity in a patient comprising:

obtaining a sample containing cells from the patient;

lysing the cells to form a cell lysate;

combining the cell lysate with a labeled substrate of the fatty acid amide hydrolase, to form a reaction mixture;

incubating the reaction mixture under conditions sufficient to allow a fatty acid amide hydrolase present in the cell lysate to hydrolyze the labeled substrate, thereby forming at least one labeled hydrolysis product;

contacting the incubated reaction mixture with a selective binding material;
wherein the selective binding material binds either the labeled substrate or a labeled hydrolysis product, but not both, thereby forming a bound labeled complex;

separating the bound labeled complex from the incubated reaction mixture;

determining an amount of labeled substrate hydrolyzed, or labeled hydrolysis product formed, thereby indicating the fatty acid amide hydrolase activity of the sample; and

comparing the activity of the sample from the patient with the activity of a to a predetermined value for activity, to determine if the patient has altered fatty acid amide hydrolase activity relative to the predetermined value for activity.

46. The method of claim 45 wherein said patient is female.

47. The method of claim 46 wherein the female is pregnant or is seeking fertility treatment.

48. The method of claim 45 wherein the sample comprises blood, tissue or body fluid.

49. The method of claim 46 wherein the sample comprises lymphocytes.

50. The method of claim 45 wherein the cells are homogenized.

51. The method of claim 45 wherein the fatty acid amide hydrolase activity present in the cell lysate is partially or substantially purified from the sample.

52. The method of claim 45 wherein the predetermined determined value is from a control assay, a prior or subsequent sample from the patient, a sample from a normal individual, a sample from another patient, a standard FAAH, or a predetermined value.

Fig FAAH Nucleic Acid Sequence (SEQ ID NO:1)

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cggtcctcgg tgggagatca tgggtgcagga agaactgtgg gctgcgttct
ccggcccttc cggggttgcc ctggcctgct gcttggtggc agcggccttg
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Pig FAAH Amino Acid Sequence (SEQ ID NO:2)

MVQEELWAAF	SGPSGVALAC	CLVAAALALR	WSSRRMARGA	AARARQRQQA
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LQKAWEVNRG	TNCVTTYLAD	CEAQLCQAPG	QGLLYGVPVS	LKECFSCCKGH
DSTLGLSRNQ	GTPAECDCVV	VQVLKLQGA	PFVHTNVPQS	MFSYDCSNPL
FGQTTNPWMS	SKSPGGSSGG	EGALIAAGGS	PLGLGTDIGG	SIRFPSAFCG
ICGIKPTGNR	ISKSGLKGSV	YGQVAVQLSV	GPMARDVESL	ALCLRALICE
DMFRLDPTVP	PLPFNEEVYA	SSRPLRVGY	ETDNYTMPTP	AMRRALLETK
RSLEAAGHTL	IPFLPANIPH	ALEALSTGGL	FSDGGKRLLO	NFEGDYVDSC
LGDILISILRL	PKWLKGLLAF	MLRPLLPRLA	GFLSSLRPRS	AGKLWELQHE
IEMYRHSVIA	QWRALDLDDV	LTPMLSPALD	LNAPGKATGA	VSYTLLYNCL
DFFAGVVPVT	TVTAEDEAQM	EHYKGYFGDI	WDKVVQKAMK	RSVGLPVAVQ
CVALPWQEEL	CLRFMREVER	LMAPGRQPS		

Mouse FAAH Nucleic Acid Sequence (SEQ ID NO:3)

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Mouse FAAH Amino Acid Sequence (SEQ ID NO:4)

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Human FAAH Amino Acid Sequence (SEQ ID NO:8)

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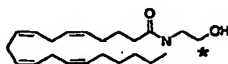
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ABSTRACT

The invention provides methods of assaying fatty acid amide hydrolase (FAAH) activity adaptable for high throughput screening. The methods provide for separating a labeled substrate from at least one labeled hydrolysis product, the separation facilitating the quantification. The invention also provides methods of identifying a compound to be tested as an inhibitor or an activator of FAAH activity through the addition of the compound to be tested to a reaction mixture and comparison of the enzyme activity in the presence and absence of the compounds to be tested. The methods are adaptable for use in detecting altered FAAH activity in patients, for example, those at risk for *in vitro* fertilization failure, or at risk for, or suffering, addictions.

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Step 1 : incubate ^3H -anandamide
with FAAH-containing membranes.



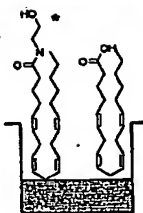
FAAH



Step 2 : load on charcoal-
filled filterplates



Step 3 : centrifuge



Step 4 : add scintillant to flow-through
and count

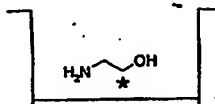


Figure 1

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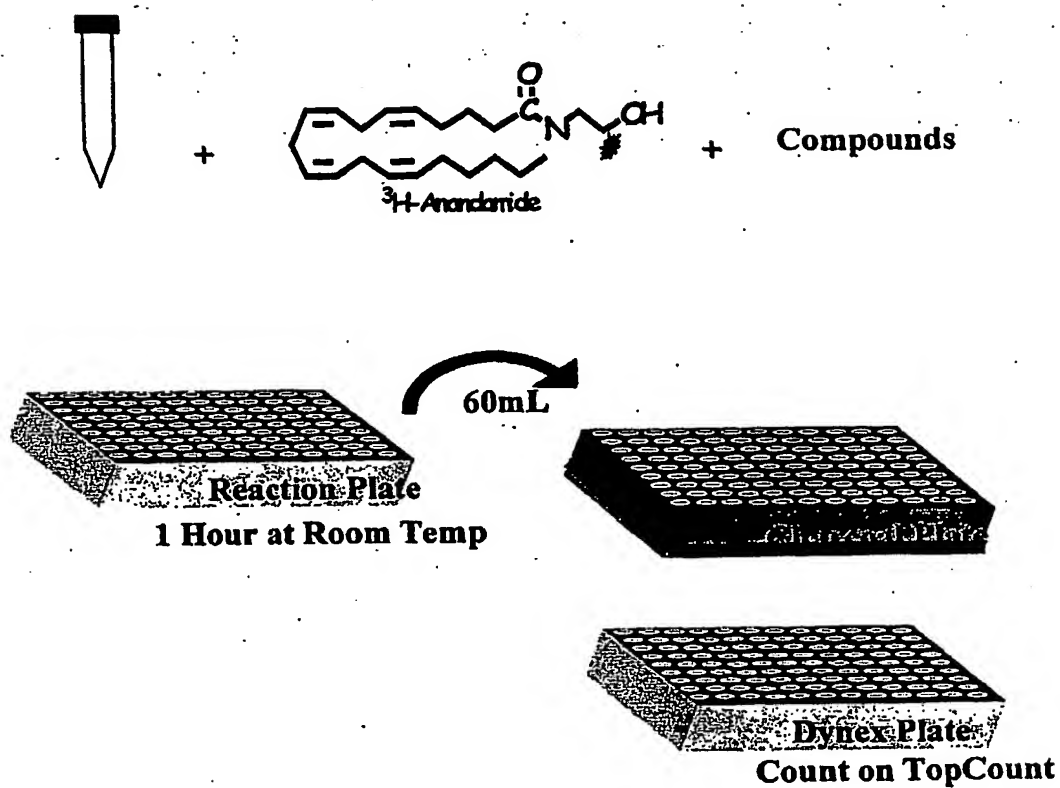


Figure 2.

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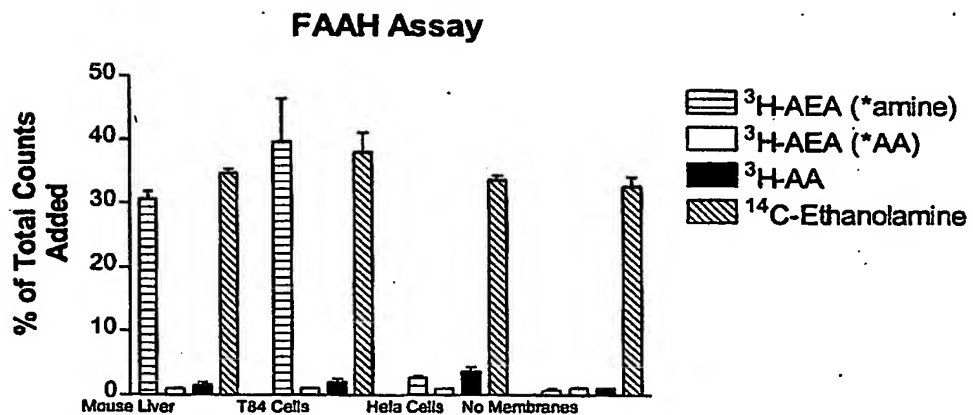


Figure 3

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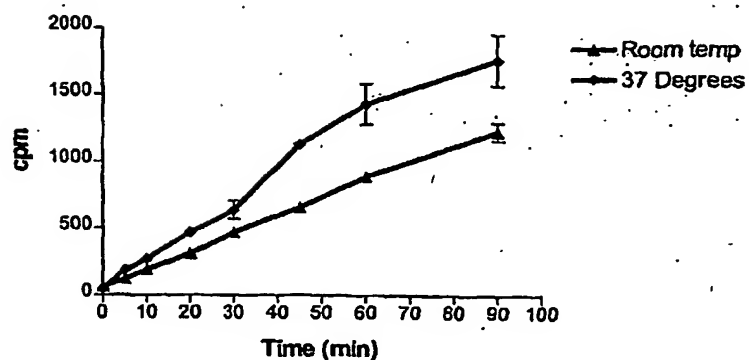


Figure 4A

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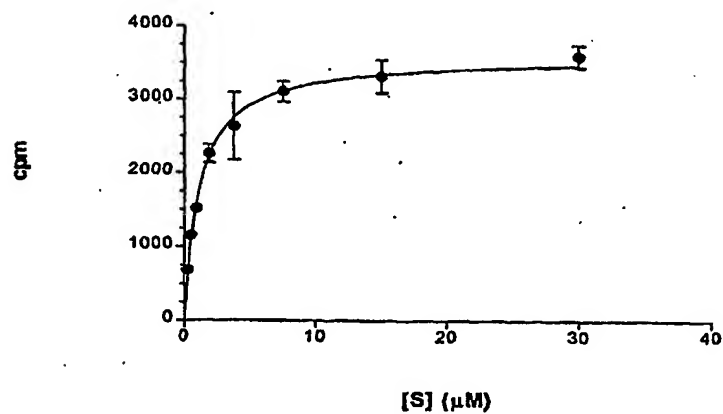


Figure 4B

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<400> 4

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Arg Ala Gly Leu Glu Thr Met Asp Lys Ala Val Gln Arg Phe Arg Leu
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Gln Asn Pro Asp Leu Asp Ser Glu Ala Leu Leu Ala Leu Pro Leu Leu
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Gln Leu Val Gln Lys Leu Gln Ser Gly Glu Leu Ser Pro Glu Ala Val
85 90 95

Leu Phe Thr Tyr Leu Gly Lys Ala Trp Glu Val Asn Lys Gly Thr Asn
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Cys Val Thr Ser Tyr Leu Thr Asp Cys Glu Thr Gln Leu Ser Gln Ala

115

120

125

Pro Arg Gln Gly Leu Leu Tyr Gly Val Pro Val Ser Leu Lys Glu Cys
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Phe Ser Tyr Lys Gly His Ala Ser Thr Leu Gly Leu Ser Leu Asn Glu
 145 150 155 160

Gly Val Thr Ser Glu Ser Asp Cys Val Val Val Gln Val Leu Lys Leu
 165 170 175

Gln Gly Ala Val Pro Phe Val His Thr Asn Val Pro Gln Ser Met Leu
 180 185 190

Ser Tyr Asp Cys Ser Asn Pro Leu Phe Gly Gln Thr Met Asn Pro Trp
 195 200 205

Lys Pro Ser Lys Ser Pro Gly Gly Ser Ser Gly Gly Glu Gly Ala Leu
 210 215 220

Ile Gly Ser Gly Gly Ser Pro Leu Gly Leu Gly Thr Asp Ile Gly Gly
 225 230 235 240

Ser Ile Arg Phe Pro Ser Ala Phe Cys Gly Ile Cys Gly Leu Lys Pro
 245 250 255

Thr Gly Asn Arg Leu Ser Lys Ser Gly Leu Lys Ser Cys Val Tyr Gly
 260 265 270

Gln Thr Ala Val Gln Leu Ser Val Gly Pro Met Ala Arg Asp Val Asp
 275 280 285

Ser Leu Ala Leu Cys Met Lys Ala Leu Leu Cys Glu Asp Leu Phe Arg
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Leu Asp Ser Thr Ile Pro Pro Leu Pro Phe Arg Glu Glu Ile Tyr Arg
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Ser Ser Arg Pro Leu Arg Val Gly Tyr Tyr Glu Thr Asp Asn Tyr Thr
 325 330 335

Met Pro Thr Pro Ala Met Arg Arg Ala Val Met Glu Thr Lys Gln Ser
 340 345 350

Leu Glu Ala Ala Gly His Thr Leu Val Pro Phe Leu Pro Asn Asn Ile
 355 360 365

Pro Tyr Ala Leu Glu Val Leu Ser Ala Gly Gly Leu Phe Ser Asp Gly
 370 375 380

Gly Cys Ser Phe Leu Gln Asn Phe Lys Gly Asp Phe Val Asp Pro Cys
 385 390 395 400

Gly Asp Leu Val Leu Val Leu Lys Leu Pro Arg Trp Phe Lys Lys
405 410 415

Leu Leu Ser Phe Leu Leu Lys Pro Leu Phe Pro Arg Leu Ala Ala Phe
420 425 430

Leu Asn Ser Met Cys Pro Arg Ser Ala Glu Lys Leu Trp Glu Leu Gln
435 440 445

His Glu Ile Glu Met Tyr Arg Gln Ser Val Ile Ala Gln Trp Lys Ala
450 455 460

Met Asn Leu Asp Val Val Leu Thr Pro Met Leu Gly Pro Ala Leu Asp
465 470 475 480

Leu Asn Thr Pro Gly Arg Ala Thr Gly Ala Ile Ser Tyr Thr Val Leu
485 490 495

Tyr Asn Cys Leu Asp Phe Pro Ala Gly Val Val Pro Val Thr Thr Val
500 505 510

Thr Ala Glu Asp Asp Ala Gln Met Glu His Tyr Lys Gly Tyr Phe Gly
515 520 525

Asp Met Trp Asp Asn Ile Leu Lys Lys Gly Met Lys Lys Gly Ile Gly
530 535 540

Leu Pro Val Ala Val Gln Cys Val Ala Leu Pro Trp Gln Glu Glu Leu
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Arg Pro Ser

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<212> DNA
<213> rattus rattus

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Arg Ala Ser Leu Glu Thr Met Asp Lys Ala Val Gln Arg Phe Arg Leu
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Gln Asn Pro Asp Leu Asp Ser Glu Ala Leu Leu Thr Leu Pro Leu Leu
 65 70 75 80

Gln Leu Val Gln Lys Leu Gln Ser Gly Glu Leu Ser Pro Glu Ala Val
 85 90 95

Phe Phe Thr Tyr Leu Gly Lys Ala Trp Glu Val Asn Lys Gly Thr Asn
 100 105 110

Cys Val Thr Ser Tyr Leu Thr Asp Cys Glu Thr Gln Leu Ser Gln Ala
 115 120 125

Pro Arg Gln Gly Leu Leu Tyr Gly Val Pro Val Ser Leu Lys Glu Cys
 130 135 140

Phe Ser Tyr Lys Gly His Asp Ser Thr Leu Gly Leu Ser Leu Asn Glu
 145 150 155 160

Gly Met Pro Ser Glu Ser Asp Cys Val Val Val Gln Val Leu Lys Leu
 165 170 175

Gln Gly Ala Val Pro Phe Val His Thr Asn Val Pro Gln Ser Met Leu
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Ser Phe Asp Cys Ser Asn Pro Leu Phe Gly Gln Thr Met Asn Pro Trp
 195 200 205

Lys Ser Ser Lys Ser Pro Gly Gly Ser Ser Gly Gly Glu Gly Ala Leu
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Ile Gly Ser Gly Gly Ser Pro Leu Gly Leu Gly Thr Asp Ile Gly Gly
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Ser Ile Arg Phe Pro Ser Ala Phe Cys Gly Ile Cys Gly Leu Lys Pro
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Thr Gly Asn Arg Leu Ser Lys Ser Gly Leu Lys Gly Cys Val Tyr Gly
 260 265 270

Gln Thr Ala Val Gln Leu Ser Leu Gly Pro Met Ala Arg Asp Val Glu
275 280 285

Ser Leu Ala Leu Cys Leu Lys Ala Leu Leu Cys Glu His Leu Phe Thr
290 295 300

Leu Asp Pro Thr Val Pro Pro Leu Pro Phe Arg Glu Glu Val Tyr Arg
305 310 315 320

Ser Ser Arg Pro Leu Arg Val Gly Tyr Tyr Glu Thr Asp Asn Tyr Thr
325 330 335

Met Pro Ser Pro Ala Met Arg Arg Ala Leu Ile Glu Thr Lys Gln Arg
340 345 350

Leu Glu Ala Ala Gly His Thr Leu Ile Pro Phe Leu Pro Asn Asn Ile
355 360 365

Pro Tyr Ala Leu Glu Val Leu Ser Ala Gly Gly Leu Phe Ser Asp Gly
370 375 380

Gly Arg Ser Phe Leu Gln Asn Phe Lys Gly Asp Phe Val Asp Pro Cys
385 390 395 400

Leu Gly Asp Leu Ile Leu Ile Leu Arg Leu Pro Ser Trp Phe Lys Arg
405 410 415

Leu Leu Ser Leu Leu Leu Lys Pro Leu Phe Pro Arg Leu Ala Ala Phe
420 425 430

Leu Asn Ser Met Arg Pro Arg Ser Ala Glu Lys Leu Trp Lys Leu Gln
435 440 445

His Glu Ile Glu Met Tyr Arg Gln Ser Val Ile Ala Gln Trp Lys Ala
450 455 460

Met Asn Leu Asp Val Leu Leu Thr Pro Met Leu Gly Pro Ala Leu Asp
465 470 475 480

Leu Asn Thr Pro Gly Arg Ala Thr Gly Ala Ile Ser Tyr Thr Val Leu
485 490 495

Tyr Asn Cys Leu Asp Phe Pro Ala Gly Val Val Pro Val Thr Thr Val
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Thr Ala Glu Asp Asp Ala Gln Met Glu Leu Tyr Lys Gly Tyr Phe Gly
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Asp Ile Trp Asp Ile Ile Leu Lys Lys Ala Met Lys Asn Ser Val Gly
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Leu Pro Val Ala Val Gln Cys Val Ala Leu Pro Trp Gln Glu Glu Leu
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Gln Pro Ser

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<400> 8

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Arg Ala Gly Leu Glu Asn Met Asp Arg Ala Ala Gln Arg Phe Arg Leu
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Gln Asn Pro Asp Leu Asp Ser Glu Ala Leu Leu Ala Leu Pro Leu Pro
65 70 75 80

Gln Leu Val Gln Lys Leu His Ser Arg Glu Leu Ala Pro Glu Ala Val
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Leu Phe Thr Tyr Val Gly Lys Ala Trp Glu Val Asn Lys Gly Thr Asn
100 105 110

Cys Val Thr Ser Tyr Leu Ala Asp Cys Glu Thr Gln Leu Ser Gln Ala
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Pro Arg Gln Gly Leu Leu Tyr Gly Val Pro Val Ser Leu Lys Glu Cys
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Phe Thr Tyr Lys Gly Gln Asp Ser Thr Leu Gly Leu Ser Leu Asn Glu
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Gly Val Pro Ala Glu Cys Asp Ser Val Val Val His Val Leu Lys Leu
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Gln Gly Ala Val Pro Phe Val His Thr Asn Val Pro Gln Ser Met Phe
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Ser Tyr Asp Cys Ser Asn Pro Leu Phe Gly Gln Thr Val Asn Pro Trp
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 Lys Ser Ser Lys Ser Pro Gly Gly Ser Ser Gly Gly Glu Gly Ala Leu
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 Ile Gly Ser Gly Gly Ser Pro Leu Gly Leu Gly Thr Asp Ile Gly Gly
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 Thr Gly Asn Arg Leu Ser Lys Ser Gly Leu Lys Gly Cys Val Tyr Gly
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 Gln Glu Ala Val Arg Leu Ser Val Gly Pro Met Ala Arg Asp Val Glu
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 Pro His Ala Leu Glu Thr Leu Ser Thr Gly Gly Leu Phe Ser Asp Gly
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 His Glu Ile Glu Val Tyr Arg Lys Thr Val Ile Ala Gln Trp Arg Ala
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Tyr Asn Cys Leu Asp Phe Pro Ala Gly Val Val Pro Val Thr Thr Val
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Thr Ala Glu Asp Glu Ala Gln Met Glu His Tyr Arg Gly Tyr Phe Gly
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Asp Ile Trp Asp Lys Met Leu Gln Lys Gly Met Lys Lys Ser Val Gly
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Leu Pro Val Ala Val Gln Cys Val Ala Leu Pro Trp Gln Glu Glu Leu
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Cys Leu Arg Phe Met Arg Glu Val Glu Arg Leu Met Thr Pro Glu Lys
565 570 575

Gln Ser Ser